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Advances in Brief

Aberrant Methylation of the Estrogen Receptor and E-Cadherin 5′ CpG Islands Increases with Malignant Progression in Human Breast Cancer[1]

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Abstract

Loss of expression for both the *estrogen receptor-α* and *E-cadherin* genes has been linked to disease progression in human ductal breast carcinomas and has been associated with aberrant 5′ CpG island methylation. To assess when, during malignant progression, such methylation begins and whether such methylation increases with advancing disease, we have surveyed 111 ductal carcinomas of the breast for aberrant methylation of the estrogen receptor- α and E-cadherin 5′ CpG islands. Hypermethylation of either CpG island was evident prior to invasion in ~30% of ductal carcinoma *in situ* lesions and increased significantly to nearly 60% in metastatic lesions. Coincident methylation of both CpG islands also increased significantly from ~20% in ductal carcinoma *in situ* to nearly 50% in metastatic lesions. Furthermore, in all cases, the pattern of methylation displayed substantial heterogeneity, reflecting the well-established, heterogeneous loss of expression for these genes in ductal carcinomas of the breast.

Introduction

Human breast carcinomas most frequently evolve from the epithelial lining of the terminal mammary ducts as DCIS[3] that may progressively become invasive and ultimately metastatic (1). The transformation of normal mammary epithelial cells into a carcinoma and the subsequent progression to invasion and metastasis involve the accumulation of numerous genetic "hits," including the activation or amplification of dominant oncogenes and the deletion or inactivating mutation of key tumor suppressor genes (2). It has recently become evident that tumor suppressor genes may also be transcriptionally silenced in association with aberrant promoter-region CpG island methylation (3, 4).

The *ERα* gene and the *E-cad* gene have been implicated frequently in the initiation and/or progression of human breast cancer. Loss of expression of either gene has been associated with poorly differentiated tumors and poorer prognosis (5–10). Furthermore, several studies have reported an association between E-cad and ER expression in breast tumors (7, 9, 10). In the case of E-cad, classical mutations and deletions may play a role in loss of gene expression (11, 12). However, loss of E-cad expression, as well as loss of ER expression, has also been associated with aberrant 5′ CpG island methylation in breast cancer cell lines and primary human breast tumors (13–18). It is currently unclear when, during malignant progression of ductal breast carcinoma, aberrant methylation of these CpG islands begins and whether the incidence of such methylation tracks with advancing disease for either or both genes. Therefore, we have evaluated a total of 111 ductal breast carcinomas for the incidence of CpG island methylation for these two key suppressor genes in *in situ*, invasive, and metastatic lesions. Our results indicate that the aberrant methylation of either CpG island begins before invasion and increases with metastatic progression. Coincident methylation of both CpG islands also increases with progression, suggesting that the malignant progression of ductal breast carcinoma involves the accumulation of multiple epigenetic "hits."

Materials and Methods

Tissue Samples. A total of 111 human breast tumor samples identified as DCIS, IDC, and LA/MDC were obtained from the Department of Pathology at Johns Hopkins University School of Medicine and from the Department of Pathology at Vanderbilt University Hospital. Seventy-five % of the LA/MDC samples were derived from lymph nodes, whereas the remaining 25% consisted of samples from a variety of sites including the chest wall, bone, and lung. Two cases of recurrent breast cancer after lumpectomy were also included. In the case of DCIS, samples were carefully microdissected prior to DNA isolation to avoid sample contamination with other cells. A portion of these tumors had been analyzed previously for E-cad methylation (18). The preliminary results of that study prompted us to expand the tumor sample pool and to include analysis of a second gene (ER). None of the results for ER methylation in this tumor set have been reported previously.

Cell Lines. Two human breast cancer cell lines were used as controls for methylation assays. MCF-7 cells express both ER and E-cad, and the CpG islands of both genes are unmethylated in this cell line. The MDA-MB-231 cell line exhibits extensive methylation of the *ER* and *E-cad* gene CpG islands, and the cells lack expression of the two genes at both the mRNA and protein level (13, 14, 16, 17). The cell lines were routinely maintained as described previously (13).

DNA Isolation. DNA was isolated from the tissues and cell lines as described previously (14, 16). DNA samples were labeled with a coded identification number so that MSP analysis could be performed and analyzed without knowledge of the sample origin.

MSP. ER and E-cad 5′ CpG island MSP was performed on sodium bisulfite-treated DNA as described previously (15, 17). The ER primers (primer set #5; Ref. 15) target a region of the gene about 400 bp downstream from the transcription start site near a *Not*I site. MSP primers spanning the transcription start site of E-cad were described previously as Island 3 (17). Earlier studies showed that methylation in the regions targeted by these primer sets correlated best with loss of gene expression (15, 17). A fraction of the tumor samples in the current study were also analyzed with additional MSP primer sets for the two genes to verify the density of CpG island methylation in these tumors. For many samples, the methylation status of ER and E-cad was assessed concurrently by including primers for both genes in the same reaction (termed duplex PCR).

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[3] The abbreviations used are: DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; E-cad, E-cadherin; MSP, methylation-specific PCR; IDC, invasive ductal carcinoma; MDC, metastatic ductal carcinoma; LA, locally advanced.

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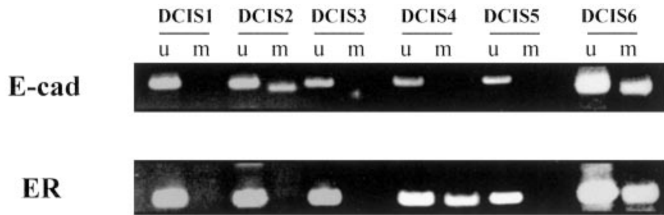

Fig. 1. MSP analysis of the E-cad and ER CpG islands in human breast cancers (DCIS). MSP was used to assess the methylation status of each CpG island. Representative results from six DCIS lesions are shown. The two genes were analyzed concurrently by performing duplex PCR reactions that contained primers for both islands. *u,* primers specific for unmethylated DNA; *m,* primers specific for methylated DNA.

Statistical Analysis. Any tumor sample that reliably yielded a PCR product in the methylated reaction visible by ethidium bromide staining was considered positive for CpG methylation. The Mantel-Haenszel χ^2 test for trend was applied to 3-by-2 tables of tumor type *versus* methylation (yes/no) to assess the change in percentage of methylation with increasing tumor progression. Then each pair of tumor types was compared using logistic regression. Significance was set at $P < 0.05$.

Results and Discussion

The ER and the homotypic cell:cell adhesion molecule, E-cad, both play a role in maintaining the normal differentiated state of the mammary gland epithelium (6, 19). Loss of the ER during breast cancer progression is associated with poorer histological differentiation, higher growth fraction, and poorer clinical outcome and may represent a key mechanism facilitating hormone resistance (5, 20). Similarly, loss of E-cad expression has been repeatedly associated with loss of differentiation, increased invasive and metastatic potential, and decreased patient survival (6, 9, 11, 21). The transcriptional silencing of both ER and E-cad in human breast cancer has been associated with aberrant promoter-region CpG island hypermethylation. In addition, treatment of human breast cancer cell lines lacking ER and/or E-cad with DNA methyltransferase inhibitor (5-deoxyazacytidine) elicits CpG island demethylation and re-expression of E-cad and ER protein, thereby indicating that aberrant methylation of these CpG islands plays a substantial role in suppressing transcription of these two key suppressor genes in breast cancer cells (16, 22).

Because expression of both ER and E-cad is lost in association with aberrant 5′ CpG island methylation during breast tumorigenesis, we sought to define the stage of breast tumor progression at which the hypermethylation of these two CpG islands begins and whether such methylation tracks with advancing disease. We analyzed a total of 111 ductal breast carcinomas comprised of *in situ* lesions (DCIS), invasive, and metastatic cancers by MSP (23).

The Incidence of CpG Island Methylation Increases with Tumor Progression. MSP has been used previously to detect aberrant DNA methylation of several genes, including *ER* and *E-cad,* in human cancers (15, 17, 22). Neither gene is methylated in normal breast epithelia (13–17). However, methylation of the two CpG islands was evident in all tumor stages and showed remarkably similar increases during progression from DCIS to metastatic tumors. Methylation of the *ER* gene was evident in 34% (12 of 35) of DCIS lesions, whereas E-cad methylation was evident in 31% (11 of 35). Coincident methylation was present in only 21% of these DCIS lesions. (Fig. 1 and summarized in Table 1). In invasive and metastatic ductal carcinomas (IDC or MDC), the incidence of methylation markedly increased relative to the DCIS lesions. Twenty-five of 48 (52%) IDC samples showed methylation of the ER or E-cad 5′ CpG island (Fig. 2; Table 1). Of these 48 samples, 18 (38%) showed distinct, coincident methylation of both CpG islands. Of the locally advanced and metastatic tumor samples, nearly 60% exhibited methylation for each of the CpG islands (Fig. 2; Table 1), whereas coincident methylation of both CpG islands was apparent in 50% (14 of 28) of these samples.

These data indicate that the epigenetic inactivation of either gene may occur early, prior to invasion, but increases as cells acquire invasiveness and metastatic potential. The Mantel-Haenzael χ^2 test for trend demonstrated that the trend toward increased methylation during progression was statistically significant for each gene ($P < 0.05$; Table 1). Furthermore, pair-wise comparison of the three tumor stages demonstrated that the incidence of methylation in metastatic tumors was significantly higher than in DCIS for both ER (odds ratio, 2.96; $P = 0.039$) and E-cad (odds ratio, 3.37; $P = 0.022$). The incidence of methylation in IDC samples was not statistically different from the other two categories, however.

The trend toward increasing coincident methylation of the two genes during progression was also statistically significant ($P = 0.013$;

Table 1 *Incidence of CpG island methylation for ER and E-cad genes in human breast tumors*

Tumor type	% ER methylation	% E-cad methylation	% ER and E-cad methylation	% ER or E-cad methylation
All	49% (54/111)	48% (53/111	35% (39/111)	61% (68/111)
DCIS	34% (12/35)	31% (11/35)	21% (7/35)	46% (16/35)
IDC	52% (25/48)	52% (25/48)	38% (18/48)	67% (32/48)
LA/MDC	61% (17/28)	61% (17/28)	50% (14/28)	71% (20/28)
P (trend) M-H[a] χ^2	0.034	0.019	0.013	0.032

[a] M-H, Mantel-Haenszel.

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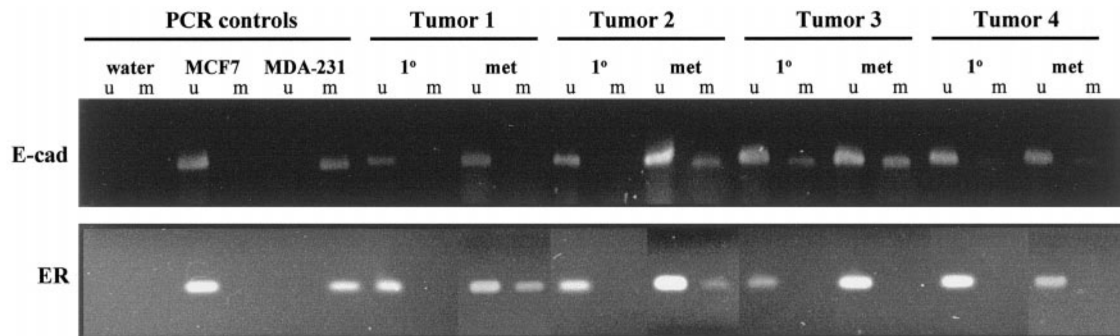

Fig. 2. MSP analysis of the E-cad and ER CpG islands in human breast cancers (IDC and MDC). Representative results from four primary (*1°*)-metastatic (*met*) pairs are shown. MSP reactions for E-cad and ER were run and analyzed separately. Metastatic sites were as follows: *1*, bone; *2*, chest wall; *3*, axillary lymph node; *4*, supraclavicular lymph node. *u,* primers specific for unmethylated DNA; *m,* primers specific for methylated DNA. Water served as a negative control, and DNA from MCF-7 and MDA-MB-231 cells served as positive controls for the unmethylated and methylated reactions, respectively.

Table 1). Thus, the frequency of coincident methylation of both CpG islands increases with advancing disease, suggesting that malignant progression of ductal breast carcinoma involves the accumulation of multiple epigenetic "hits." However, it is important to note that the similarity in the trends for ER and E-cad methylation was not attributable to complete coincidence of methylation for the two genes. At every stage of progression, the rate of coincident methylation was lower than the incidence of methylation for each individual gene (Table 1). Overall, ~25% of the samples analyzed showed methylation of either ER or E-cad, but not both. These results imply that aberrant methylation of these CpG islands does not simply reflect a generalized increase in CpG island methylation but may reflect a more specific selection process targeting key suppressor genes.

CpG Island Methylation Is Heterogeneous in Breast Tumors. In all samples harboring methylation, unmethylated alleles were invariably also evident (Figs. 1 and 2). For the IDC and LA/MDC samples, which were not microdissected, these unmethylated alleles may reflect the contribution from normal cells in the sample. Alternatively, these alleles may be derived from cancer cells that harbored only unmethylated copies of the E-cad and ER CpG islands. However, this same heterogeneous pattern was evident in the methylated DCIS samples, which were carefully microdissected, suggesting that methylation of these CpG islands in these tumors is heterogeneous. Interestingly, expression studies have routinely revealed that the loss of both E-cad and ER exhibits distinct heterogeneity in ductal breast carcinomas (6, 9, 10, 24). In addition our earlier studies have demonstrated that heterogeneity of both ER (15) and E-cad (18) methylation is associated with heterogeneity of protein expression. Limitations in our ability to recover the tissue specimens associated with these DNA samples (especially those derived from *in situ* lesions) precluded a simultaneous evaluation in this study. However, it seems likely that the heterogeneous patterns of CpG island methylation parallel the heterogeneous loss of E-cad and ER expression in these tumors.

In summary, these data indicate that the malignant progression of human ductal breast carcinomas involves a heterogeneous pattern of methylation for both the ER and E-cad 5′ CpG islands that begins prior to the acquisition of invasiveness and increases for each CpG island with advancing disease. In the case of E-cad, these results are particularly striking because loss of E-cad expression is generally associated with the acquisition of invasive or metastatic potential rather than the earlier stages of tumorigenesis. Finally, the increase in the coincident methylation of both CpG islands suggests that malignant progression of human breast cancer involves not only the well-documented accumulation of genetic "hits" but also an accumulation of epigenetic "hits" that contribute to the diminished expression of key tumor suppressor genes like *ER* and *E-cad*.

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